Award Number: W81XWH-15-1-0639

TITLE: The Role of BMI1 in CRPC

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REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Annual	21 Sep 2016 - 20 Sep 2017

4. TITLE AND SUBTITLE

The Role of BMI1 in CRPC

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0639

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our preliminary data strongly suggest that BMI1 is a master regulator of castration-resistant prostate cancer (CRPC) progression. Our objective is to determine how BMI interacts with epigenetic complexes and with AR to regulate tumor suppressor gene expression. We aim to identify novel binding partners and regulators of oncogene expression, which will lead to a better understanding of AR signaling and dysfunction. Specifically, we will identify how BMI1 and PRC1 proteins mediate their oncogenic functions by recruiting AR and distinct binding partners to promote castration-resistance of PCa. Furthermore, we will evaluate the therapeutic efficacy of targeting BMI1 and of combinational targeting of BMI1 and AR in castration-resistant prostate cancer. During the first year of this project, we discovered that BMI1 directly binds to Androgen Receptor and prevents it from MDM2-mediated protein degradation. We further demonstrated that inhibiting BMI1 decreased prostate cancer tumor growth in VCaP murine xenograft.

15. SUBJECT TERMS: **Polycomb, BMI1, Androgen Receptor, ubiquitination, PRC1, castration-resistant prostate cancer**

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Each year, over 240,000 American men are diagnosed with prostate cancer (PCa). B lymphoma Mo-MLV insertion region 1 homolog (BMI1) have been shown associating with metastatic prostate cancer by cDNA microarray analyses and tissue microarray analysis. BMI1 is an epigenetic component of a Polycomb Repressive Complex 1 (PRC1), maintaining gene repression. We have demonstrated that BMI1 promotes prostate cancer progression by repressing multiple tumor suppressors. However, its precise role in castration-resistant prostate cancer (CRPC) remains unclear. Our preliminary data strongly suggest that BMI1 is a master regulator of castration-resistant prostate cancer (CRPC) progression. Our objective is to determine how BMI interacts with epigenetic complexes and with AR to regulate tumor suppressor gene expression. We aim to identify novel binding partners and regulators of oncogene expression, which will lead to a better understanding of AR signaling and dysfunction. Specifically, we will identify how BMI1 and PRC1 proteins mediate their oncogenic functions by recruiting AR and distinct binding partners to promote castration-resistance of PCa. Furthermore, we will evaluate the therapeutic efficacy of targeting BMI1 and of combinational targeting of BMI1 and AR in castration-resistant prostate cancer.

KEYWORDS

BMI1, Prostate Cancer, Polycomb Repressive Complex, Androgen Receptor, Castration-Resistant Prostate Cancer, small molecule inhibitor

ACCOMPLISHMENTS

A. What were the major goals of the project?

	Months	Percentage of completion
Major Task 1: to elucidate the mechanism by which BMI1 interacts with AR and recruits AR	1-24	100%
Major Task 2: to dissect how BMI1 plays its role in androgen signaling	1-36	70%
Major Task 3: to evaluate BMI1 as a therapeutic target for advanced prostate cancer patient treatment.	1-36	70%
Milestone(s) Achieved: discovery of critical domains for AR and PRC1 protein interactions; determination of binding affinity of AR and PRC1, and set-up of a high-throughput platform for small molecule inhibition screening	24	90%
Milestone(s) Achieved: identification and characterization of novel binding partners and downstream targets of BMI1 and AR in androgen-dependent and -independent PCa cells, in the presence and absence of androgen.	36	80%
Milestone(s) Achieved: evaluation of BMI1 as a therapeutic target for CRPC patients and rationale for combinatorial targeting of AR and BMI1 in clinic trials; publication of 1-2 peer reviewed papers	36	70%

B. What was accomplished under these goals?

1. **BMI1 binds to AR variants**

As reported last year, BMI1 binds to AR N-terminal domain (NTD). Since all AR variants still have NTD domain, we tested if BMI1 binds to these AR variants. Here we used AR-V7 and AR-Ve567s as examples. We overexpressed AR full-length (FL), AR-V7 or AR-Ve567s into 293T cells and then performed immunoprecipitation (IP) with anti-AR (Fig. 1A) or anti-BMI1 (Fig. 1B) and demonstrated that BMI1 interacts with full-length AR, as well as the AR variants. Further, we demonstrated the interaction between endogenous BMI1 and AR-V7 as well as AR full-length in 22RV1 cells (Fig. 1C) which harbors AR-V7 and AR-FL.

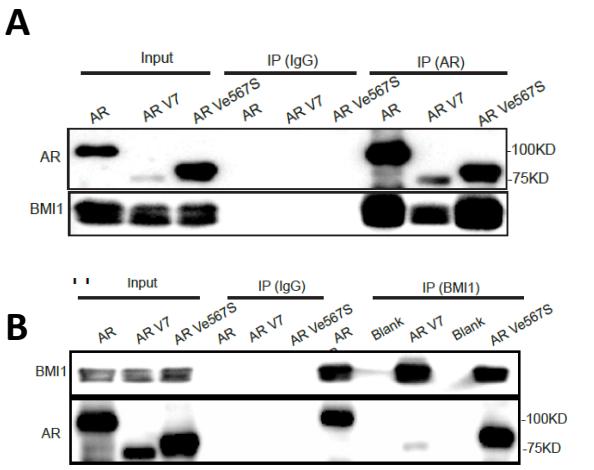

Figure 1. BMI1 binds to AR variants.

(**A, B**) Pull-down assay of HEK-293T cell lysates ectopically overexpressing AR full-length, AR-V7 or AR-Ve567s with anti-AR (**A**) or anti-BMI1 (**B**) followed by IB analysis with anti-AR and anti-BMI1 confirmed the AR variants and BMI1 interaction. (**C**) IP using 22RV1 cell lysates with anti-AR or anti-BMI1 followed by IB analysis with anti-AR and anti-BMI1 confirmed the endogenous AR-V7 and BMI1 interaction.

We generated BMI1 truncated mutants BMI1-RING and BMI1ΔRING (Fig. 2A). As previously reported, we showed that BMI1ΔRING did not bind to RING1B (Fig. 2B). However, BMI1ΔRING, as well as BMI1 full-length and MDM2 (a previously reported AR binding protein) interacted with AR, but RING1B did not interacts with AR (Fig. 2C).

2. BMI1ΔRING inhibits MDM2-mediated AR degradation.

We next performed in vitro interaction competition assays using the purified AR-NTD, MDM2 and BMI1ΔRING proteins. As shown in Fig. 2D, the presence of BMI1ΔRING disrupted the interaction between MDM2 and AR. More importantly, ectopic overexpressing RNAi-resistant BMI1ΔRING or BMI1 could completely restored the decrease of AR induced by BMI1 knockdown (Fig. 2E), demonstrating a novel PRC1-independent function of BMI1 through its ΔRING domain.

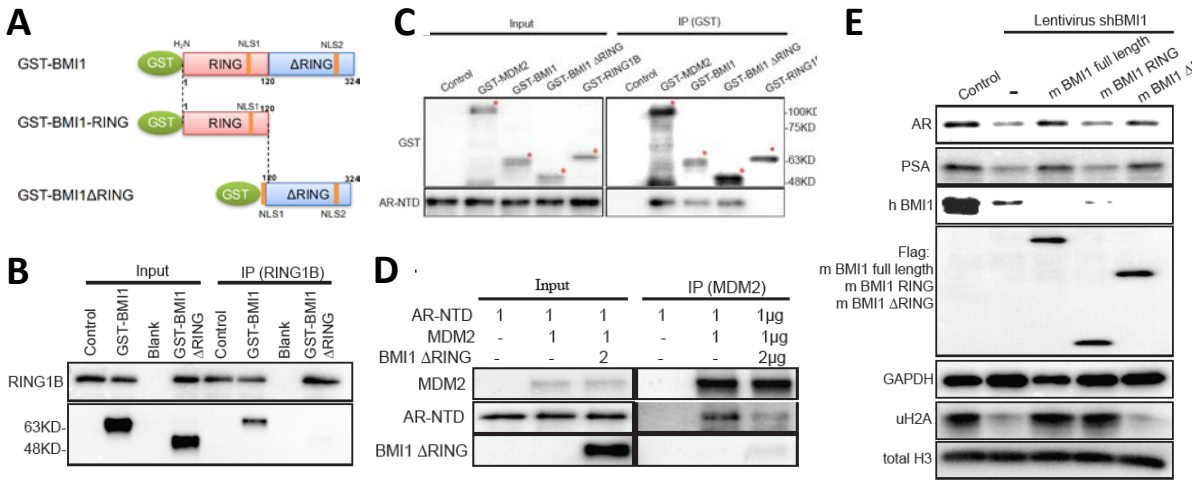

Figure 2. BMI1ΔRING regulates AR protein stability independently of PRC1 complex.

(A) Schematic of BMI1 structures. **(B)** GST-BMI1 or GST-BMI1ΔRING was incubated with RING1B for 12h followed by IPs with an anti-RING1B antibody. (**C**) His-AR-NTD was incubated at 4 ^{0}C with GST-MDM2, GST-BMI1, GST-BMI1ΔRING or GST-RING1B for 12h, followed by GST pull-down and immunoblot analysis with anti-GST and anti-AR antibodies. (**D**) Purified AR-NTD and MDM2 proteins were incubated with purified BMI1ΔRING protein at indicated concentration (0 or 2µg) at 4°C for 12h, followed by MDM2 pull-down assay. The blots were probed with anti-AR, MDM2 or -BMI1 antibodies. (**E**) C4-2 cells were infected with shBMI1 lentivirus, at 24h post infection cells were infected with flag-tagged mouse full length BMI1 lentivirus, mouse BMI1-RING lentivirus or mouse BMI1ΔRING lentivirus as indicated, cells were lysed after another 24 hours and probed by indicated antibodies. All experiments were biologically repeated at least three times. Representative images are shown.

3. BMI1 regulates AR and prostate cancer progression independent of PRC1

To better understand the functions of BMI1 in CRPC, we performed RNA-Seq analysis using BMI1 or RING1B knockdown C4-2 cells. We observed that 1,794 genes were upregulated, while 1,897 genes were downregulated by knockdown of BMI1, suggesting that BMI1 functions as a transcriptional activator along with its well-known transcriptional repression role in cancer. Intriguingly, while a majority (69.2%) of RING1B-regulated genes were also regulated by BMI1, only 39.6% of BMI1 regulated genes were also regulated by RING1B, indicating that BMI1 plays an additional role separate from its canonical functions as a component of PRC1. Gene expression profiling analysis further revealed that genes regulated by BMI1 and/or RING1B could be clustered into six groups: downregulated (group 1) or upregulated (group 4) by both BMI1 and RING1B knockdown; downregulated (group 2) or upregulated (group 5) by BMI1, but not RING1B knockdown; and downregulated (group 3) or upregulated (group 6) by RING1B, but not BMI1 knockdown (Fig 3A). We analyzed 113 AR-regulated genes derived

from cell lines, human prostate cancer, and castration-resistant prostate cancer tissues, and found their expression levels significantly dysregulated by BMI1 knockdown, but not RING1B. Gene Set Enrichment Analysis (GSEA) using androgen-induced genes further confirmed that AR-activated genes were significantly enriched in genes down regulated in BMI1 knockdown, but not RING1B knockdown (Fig. 3B). Gene expression heat maps also confirmed that AR-induced genes were significantly down regulated by BMI1 knockdown but not RING1B (Fig. 3B). Intriguingly, KEGG pathway enrichment analysis revealed that BMI1 activated genes, but not BMI1 repressed genes, were associated with protein lysine degradation, prostate cancer, and several other cancers. Importantly, survival analysis of two prostate cancer gene expression data sets both revealed that higher expression levels of BMI1 activated genes (those downregulated by BMI1 knockdown) were significantly associated with poorer disease-free survival (Figure 3C). In addition, patients with mutations or copy-number alterations of these BMI1-activated genes also had shorter disease-free survival time, compared to patients without these genomic alterations.

To further investigate how BMI1 plays its role in AR signaling, we performed ChIP-Seq analysis using anti-AR and anti-BMI1 antibodies. Our analysis revealed that AR and BMI1 were recruited to 8,442 and 2,774 genes, respectively. Intriguingly, AR was recruited to 70.9% (2.11 fold larger than random expectation) of BMI1 occupied genes (Fig. 3D) and they shared the binding sites on most of these genes (Fig. 3E), suggesting that BMI1 may directly regulate AR targets. In addition, the genes bound by BMI1 significantly overlapped with BMI1-activated genes, but not with BMI1-repressed genes, suggesting that the binding of BMI1 on chromatin is more likely to activate transcription (Fig. 3F). ChIP-qPCR analysis further confirmed that BMI1 knockdown decreased the enrichments of AR and BMI1 in upstream regions of well-known AR (Fig. 3G) and BMI1 target genes.

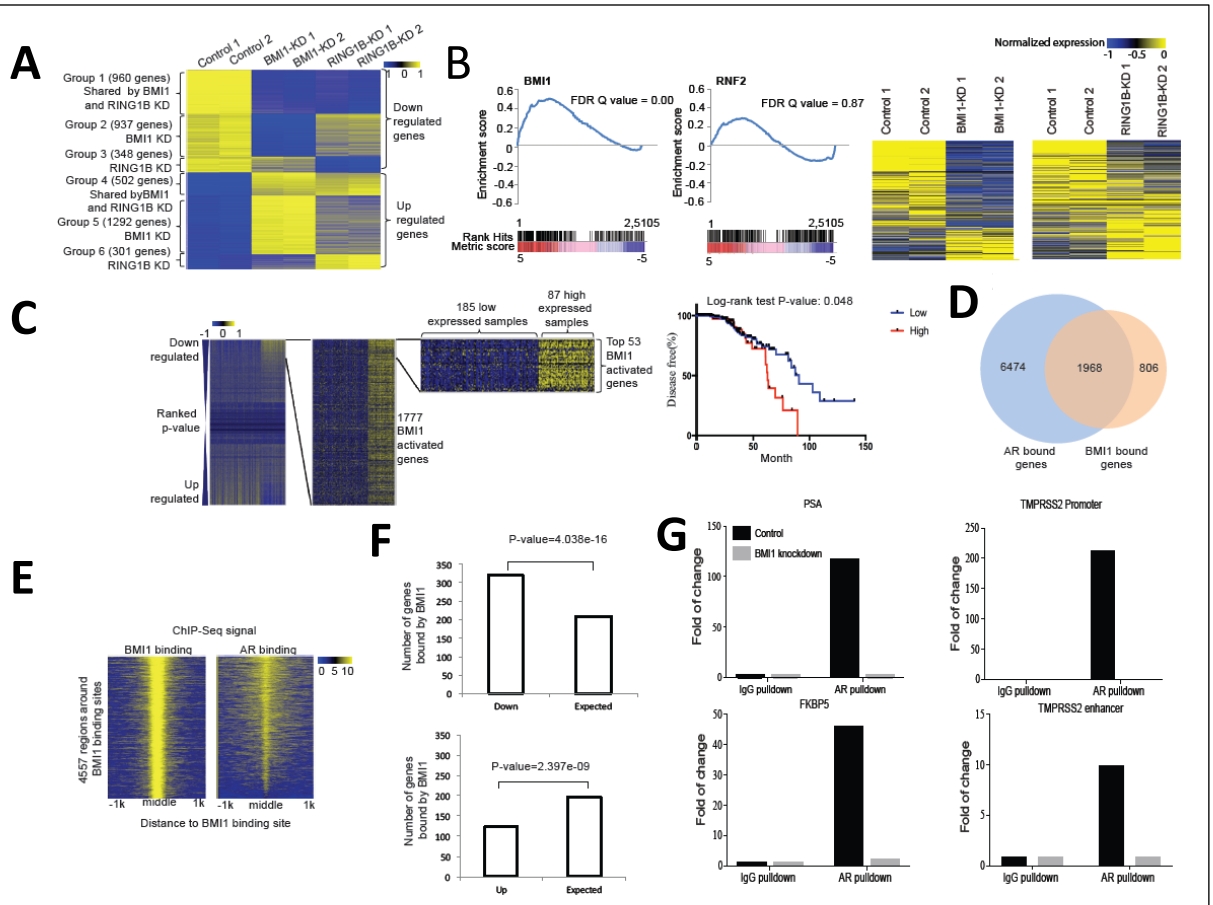

Figure 3. BMI1 regulates AR and CRPC independently of PRC1.
(A) Heat maps for expression level of genes down- or up- regulated by BMI1 or RING1B knockdown. **(B)** GSEA analysis showing enrichment level of AR-induced genes in the genes down-regulated by BMI1 or RING1B knockdown, and heat maps for expression level of the AR-induced genes**. (C)** Kaplan-Meier (KM) analysis of prostate cancer relapse based on expression level of 53 genes that were most significantly activated by BMI1 genes. TCGA gene expression data were collected from the Cbioportal database. Expression level of each gene in each sample were plotted in the heatmap. **(D)** Venn diagram showing overlap of genes bound by AR and BMI1 as determined by ChIP-Seq data. **(E)** Heatmap showing the binding density of BMI1 and AR around individual binding sites of BMI1. **(F)** Number of genes bound by BMI1 and down- or up-regulated by BMI1 knockdown. Number of genes expected by chance is also plotted. P value was calculated by Fisher's exact test. **(G)** AR and IgG ChIP was conducted in C4-2 cells transfected with shBMI1 lentivirus or vector virus for 5 days. ChIP-qPCR was conducted using gene-specific primers.

4. BMI1 inhibitor PTC209 decreased tumor progression of CRPC xenograft.

To assess the therapeutic effect of BMI1 in AR-positive PCa and CRPC, we utilized several mouse xenograft models. First, in the VCaP model we observed that, compared to vehicle control treatment, PTC209 treatment significantly inhibited tumor growth with no effect on body weight. Since our data strongly support that BMI1 regulates AR signaling in CRPC cells, and treatment of CRPC is still limited, we used a castration-resistant VCaP xenograft mouse model to evaluate therapeutic potential of BMI1 inhibition in CRPC, and PTC209 treatment significantly reduced tumor growth compared to vehicle control treatment (Fig. 4A left panel). An aggregate analysis of the initial tumor doubling time showed a significant decrease in the PTC209 treatment group compared with the vehicle group (Fig. 4A right panel). Mice bearing castration-resistant tumors demonstrated markedly faster progression of the disease compared with the treatment group. Next, we evaluated a potential synergistic effect of combination treatment with PTC209 and enzalutamide on CRPC tumor growth. While PTC209 showed similar inhibition potential as enzalutamide (Fig. 4B), combinatorial treatment of PTC209 and enzalutamide showed significantly better outcomes compared to each treatment alone and had no effect on body weight.

The rapid development of therapy resistance in prostate cancer patients subjected to enzalutamide treatment is becoming a major clinical challenge. One of the important mechanisms of enzalutamide-resistance is the increased expression of AR variants lacking the ligand binding-domain, the best characterized of which is AR-V7. To mimic enzalutamide-resistance in vivo, 20 castrated male SCID mice carrying xenografts of 22Rv1 tumors were treated with enzalutamide for 4 weeks, then sections of the biggest tumor (~1500 mm3) among these mice were transplanted to next passages of castrated male SCID mice treated with enzalutamide to maintain the enzalutamide-resistance. These mice were randomly divided into two groups when the tumors reached 100 mm3. One group was treated with PTC209 plus enzalutamide for 21 days, whereas the other groups was treated with enzalutamide alone to serve as a control (Fig. 4C). Compared to the control, PTC209 treatment significantly decreased enzalutamide-resistant CRPC tumor growth (Fig. 4D), which suggests that BMI1 is a novel therapeutic target for clinically enzalutamide-resistant CRPC. There was no difference in body weight between these two groups

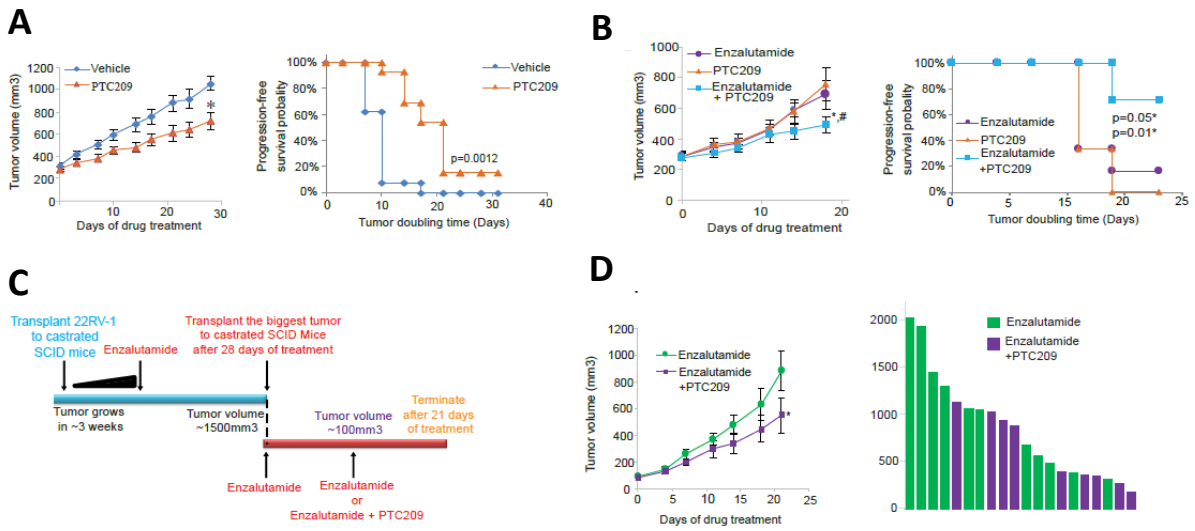

Figure 4. BMI1 inhibitor PTC-209 remarkably decreased CRPC tumor growth.

(A) Castrated mice carrying CRPC xenograft received vehicle or PTC209 (60 mg/kg/day) 5 days per week. Caliper measurements were taken every 4 days to obtain (A, left panel) Mean tumor volume±SEM; (A, right panel) Kaplan-Meier survival plot compares the progression-free survival; *P<0.05 vs. Vehicle. Castrated mice carrying CRPC xenograft received enzalutamide (10 mg/kg/day), PTC209 (60 mg/kg/day), or PTC209 (60 mg/kg/day) + enzalutamide (10 mg/kg/day) 5 days per week. Caliper measurements were taken every 4 days to obtain (**B**, left panel) Mean tumor volume±SEM; (**B**, right panel) Kaplan-Meier survival plot compares the progression-free survival; *P<0.05, PTC209+Enzalutamide vs. Enzalutamide; #P<0.05, PTC209+Enzalutamide vs. PTC209. (**C**) Enzalutamide-resistant CRPC mouse xenograft experimental design is illustrated. Castrated mice carrying enzalutamide-resistant CRPC xenograft received enzalutamide (10 mg/kg/day), enzalutamide (10 mg/kg/day) + PTC209 (60 mg/kg/day) (10 mg/kg/day) 5 days per week; (**D**, left panel) Mean tumor volume±SEM; (**D**, right panel) Waterfall plot of tumor volume response are shown. *P<0.05, Enzalutamide+PTC209 vs. Enzalutamide. VCaP cells were implanted into left flanks of 6-week old male SCID mice. Tumors upon reaching volume of 100mm^3 were subjected to treatment with vehicle control (CTRL, n=13), PTC209 (60mg/kg, n=11) alone or MDV3100 (10 mg/kg, n=12) I.P. 5 times per week. Mice were closely monitored and weighed every day. *p<0.05 (student t-test, between vehicle and PTC209).

C. What opportunities for training and professional development has the project provided?

Award/grant

2017-2020, DoD PCRP Idea-Development Award (Established Investigator Option)
2017-2022, R01 (NCI)

National/International conferences attended

Oct. 26-29, 2016, 23rd Annual Prostate Cancer Foundation Scientific Retreat, Carlsbad, CA

April 1- 5, 2017, AACR Annual Meeting, Washington DC

Jun 29-Jul 2, the 16th SCBA International Symposium, Hangzhou, China

D. How were the results disseminated to communities of interest?

Nothing to Report.

E. What do you plan to do during the next reporting period to accomplish the goals?

We are continuously working on this project and pursue the aims.

Besides the xenograft assays reported here (Fig. 4), we are evaluated if BMI1 inhibitor PTC-209 could inhibit the tumor growth using the patient-derived xenografts (PDXs). In addition, we are evaluating if the newly developed BMI1 inhibitor PTC596, which is in the phase I clinic trial, could inhibit tumor growth of CRPC and drug-resistant CRPC using the PDX models.

IMPACT

A. What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report

B. What was the impact on other disciplines?
Nothing to Report

C. What was the impact on technology transfer?
Nothing to Report

D. What was the impact on society beyond science and technology?
Nothing to Report

CHANGES/PROBLEMS

A. Changes in approach and reasons for change
Nothing to Report

B. Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

C. Changes that had a significant impact on expenditures
Nothing to Report

D. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

E. Significant changes in use or care of human subjects
Nothing to Report

F. Significant changes in use or care of vertebrate animals.
Nothing to Report

G. Significant changes in use of biohazards and/or select agents
Nothing to Report

PRODUCTS:

A. Publications, conference papers, and presentations

a. Journal publications.

Two manuscripts are under review/revision

b. Books or other non-periodical, one-time publications.

Nothing to Report

c. Other publications, conference papers, and presentations.

Poster presentation

1) Sen Zhu, Jungsun Kim, Bingnan Gu, Weihua Jiang, Lin Yan, Ladan Fazli, Jonathan Zhao, Xuesen Dong, Jindan Yu, Qi Cao. A Novel Role of BMI1 in Androgen Receptor Pathway. 23rd Prostate Cancer Foundation Annual Scientific Retreat, Oct 26-29, 2016, Carlsbad, CA

Oral presentations

Jun 1, 2016	The Coordinated Regulation of Polycomb Group Proteins in Cancer, Department of Pharmacology, University of Maryland School of Medicine, Baltimore, MD
Jun 13, 2017	A Novel Role for BMI1 in Prostate Cancer, Department of Urology, Peking University First Hospital, Beijing, China
Jun 23, 2017	Epigenetic Regulation of Prostate Cancer, Center for Regeneration Medicine and Stem Cell Engineering, Sun Yat-sen University Zhangshan School of Medicine, Guangzhou, China
Jul 1, 2017	A Novel Role for BMI1 in Prostate Cancer, the 16th SCBA International Symposium, Hangzhou, China
Sep 28, 2017	A Novel Role for BMI1 in Prostate Cancer, Annual Summit on Cell Signaling and Cancer Therapy, Chicago, IL

B. **Website(s) or other Internet site(s)**

Nothing to Report

C. **Technologies or techniques**

Nothing to Report

D. **Inventions, patent applications, and/or licenses**

Nothing to Report

E. **Other Products**

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

A. What individuals have worked on the project?

Name:	*Qi Cao*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	*Conceive the idea, lead the project, design experiments and analyze the data*
Funding Support:	*DoD PCRP IDA, Prostate Cancer Foundation, American Cancer Society, Start-up*

Name:	*Sen Zhu*
Project Role:	*Post-Doctoral*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	*Perform major experiments and analyze the data*
Funding Support:	*DoD PCRP IDA, Prostate Cancer Foundation, American Cancer Society, Start-up*

Name:	*Weihua Jiang*
Project Role:	*Post-Doctoral*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Perform xenograft experiments and analyze the data*
Funding Support:	*Prostate Cancer Foundation, American Cancer Society, Start-up*

Name:	*Lin Yan*

Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	*Help Drs. Zhu and Jiang perform xenograft experiments and molecular and cellular assays, and analyze the data*
Funding Support:	*Prostate Cancer Foundation, American Cancer Society, Start-up*

B. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

C. **What other organizations were involved as partners?**

1. Collaborator Names: Jindan Yu and Jonathan Zhao
   Organization Name: Northwestern University Feinberg School of Medicine
   Location of Organization: Chicago, IL, USA
   Partner's contribution to the project: **Collaboration**

2. Collaborator Names: Xuesen Dong and Ladan Fazli
   Organization Name: Vancouver Prostate Centre and Department of Urologic Sciences, University of British Columbia
   Location of Organization: Vancouver, BC, Canada V6H 3Z6
   Partner's contribution to the project: **Collaboration, Facilities**

3. Collaborator Names: Ming Hung
   Organization Name: Department of Urology, University of Washington
   Location of Organization: Seattle, WA, USA
   Partner's contribution to the project: **Collaboration**

4. Collaborator Names: Xiaobing Shi
   Organization Name: Department of Epigenetics, MD Cancer Center
   Location of Organization: Houston, TX, USA
   Partner's contribution to the project: **Collaboration**

5. Collaborator Names: Kaifu Chen, Dongyu Zhao, Jie Lu
   Organization Name: Center for Cardiovascular Sciences, Houston Methodist Research Institute
   Location of Organization: Houston, TX, USA
   Partner's contribution to the project: **Collaboration**